Title of the project

"Promoting β-Catenin degradation by identifying small molecule from protein-protein interface to prevent its interaction with Ubiquitin Specific Proteases 7"

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Highlights:

- Ubiquitin specific proteases 7 (USP7) is the attractive therapeutic target which is involved mainly in cancer, DNA damage responses and to be more précised multiple myeloma and which has shown its interaction with human proteins, such as p53, MDM2, β-Catenin and other proteins involved in degradation pathways.

- Molecular mechanism related to USP7 is still unclear because of the trivial complexities. The understanding USP7 at structural level will be very important as it involves three internal regulatory domains namely TRAF-domain, Catalytic domain and Ubiquitin-like domains (UBL) that decides the substrate fate of either rescue or degradation.

- USP7 inhibition is important as it rescues its substrate which are required to be degraded in abnormal conditions/over-expression. Though few USP7 inhibitors are known (P5091 and P22077) with very low potency but their structural insights like binding site, binding mode, interacting key residues and interface information is still not known.

- Characterization of integral information will not only elucidate the inhibitory mechanism but also sheds light on USP7 interaction with other substrates where binding site information will help in combating its association and also discovering novel potential modulators with increased affinity and efficiency.

- Establishing the USP7 and their interacting substrates information would be the possible druggable site, rather than inhibiting USP7 directly. In this regard we are addressing the complex formation between USP7 with β-Catenin, where inhibiting USP7 will promote degradation of β-Catenin, involved in Wnt signaling pathway.

- Discovering modulator can provide a means to better understand the endogenous methods of enzyme activation and underlie allosteric mechanism, which is the primary requisite for structure-guided lead identification.

- **All together this work highlights the atomic level resolution of inhibition mechanism of USP7 and identification of novel hits to accelerate the β-Catenin degradation through peptidomimetics, fragment-based and virtual screening approaches.**

Introduction:

Ubiquitin specific proteases 7 belong to the family of DUBs (deubiquitinating enzyme) which are responsible for removing ubiquitin from the substrate and thereby rescues the protein[1]. Ubiquitin Specific Proteases 7 (USP7), is the best studied cysteine proteases in DUBs family, because of its involvement in wide range of targets and their related pathways[2-3] and mainly because of its key roles in cancer progression[4]. USP7 has been shown to be involved with many targets (p53, MDM2, TSPYL5, H2B, Chk1, Claspin, UVSSA, SCML2, DNMT1, BRCA1-A and RNF168 and RNF220)[4-15]. USP7 as a drug target has been highlighted in tumorigenesis[16,17,18] and its overexpression in prostate cancer correlates with tumor aggressiveness[18].In a recent study, USP7 has been found as an emerging target in Wnt signaling related carcinogenesis[19]. It has been proven that knockdown of USP7 in colorectal cancer (CRC) cell lines with hyperactivated Wnt signaling downregulates the activity of Wnt signaling[20]. Therefore, investigating the possibility of USP7 as a drug target in CRC therapy would be quite beneficial to come up potent inhibitor so that growth of colorectal cancer can be capped at early stage.

Aberrant activation of Wnt/β-catenin signaling is closely associated with the development of various human cancers, especially colorectal cancers (CRC)[21]. In the absence of Wnt ligand, β-catenin in the cytosol is phosphorylated by the destruction complex, which consists of the adenomatous polyposis coli (APC), axin, glycogen synthase kinase 3β (Gsk3β), and casein kinase 1α (Ck1α)[22,23]. In the presence of Wnt ligand, the destruction complex dissociates and β-catenin in its hypophosphorylated form escapes degradation, undergoes nuclear translocation, and activates Wnt target genes, including c-myc and cyclin D1[23]. β-catenin became an important drug target after evidences have been found to support tumor growth by promoting angiogenesis in cancers[24] and also its oncogenic mutations have also been reported in APC wild-type colon cancers[25]. Also, high-level cytoplasm expression and nuclear localization of β-catenin always induces tumorigenic traits and promotes cancer cell proliferation and survival[26]. The activity of β-catenin is controlled by a large number of binding partners that affect its stability, cellular localization and transcriptional activity.

Much of the studied binding partners are USP7 and RNF220 (E3 ligase) [27-30]. Recent studies have shown that RNF220 mediates the binding of USP7 and β-catenin as RNF220 together with USP7 is responsible for deubiquitination of β-catenin where N-term domain of USP7 interacts with 315 amino acid fragment of RNF220. RNF220 and USP7 does not regulate each other in any condition

which suggests they are not effective substrates of each other. RNF220 and USP7 most likely work in complex to deubiquitinates and stabilize β-catenin, as both of these are required for the process. leading to its deubiquitination and stabilization as well[31-32.] Hence, to understand these regulatory mechanisms will be of great importance for identification of potential targets to fight against Wnt/ β-catenin-related cancers. This study will reveal out the structural aspects of the components of Wnt signaling pathway, where investigating modulators and protein-protein interface by implementing protein-ligand and peptidomimetics approaches against USP7 and USP7-RNF220 complex will elucidate deep insights in Wnt signaling pathway.

Review of Literature:

Through the course of various studies, USP's has emerged as potential therapeutic targets and amongst them USP7 has been investigated a lot due its involvement in multiple myeloma, DNA damage and colorectal cancer.

Hu et al, 2002, came up with first structural analysis of USP7, which created an exception in USP family, as it revealed that the *Apo* structure of USP7 is not functionally (catalytic domain 208-536 amino acid) active and highlighted that it needs C-terminal domains (563-1102 amino acid) to achieve its functional activity.

Brooks and Gu 2006, highlighted the deubiquitination activity of USP7 in p53 pathway, affecting both MDM2 and p53 due to which it leads to multiple and hence the need of inhibiting USP7 was emerging.

Fasen et. al 2011, highlighted the mechanistic reasons that can activate USP7. Following up Hu et al study, he showed that UBL45 (Ubiquitin like domain) of USP7 is important for its activity as it interacts with Switching loop localized in catalytic domain, which causes rearrangement in catalytic triad (C223, H464 and D481) residues consequently making USP7 active.

D. Chauhan et. al. 2012, brought the first specific and selective inhibitor of USP7 named as P5091 with 4.2 micromolar potency. However, he did not mention the binding site of this molecule, which kept guessing the inhibition mechanism of USP7 and hence an upcoming co-crystal was expected.

Meanwhile **Yan Li 2017**, used P5091 as chemical tool and verified the positive role of USP7 in Wnt signaling and colorectal tumor growth and provided the proof-of-concept for the potential if inhibitors targeting USP7 in the treatment of colorectal cancer.

Turnbull et al, 2017, was the first who introduced the first co-crystal of USP7 with inhibitor displaying 58nm potency. Also, he defined the inhibition mechanism but still a co-crystal at stagnant pose cannot give all answers.

Gaps Identified

1) Although, previous studied have come up with potential inhibitors but still their mechanism of inhibition is still not understood.
2) It is still in mystery that what activates USP7 :: a) C-terminal domains of USP7 or b) attachment of ubiquitin to catalytic domain. An exhaustive MD simulation can be a potential answer to solve this mystery.
3) Switching loop causes rearrangement but its mechanism is not understood. How does it rearrange the catalytic and makes it catalytic competent?
4) No evidence of activation of USP7 through C-terminal tail. It is still to be uncovered.
5) The emergence of co-crystal will be very useful to develop more potential and specific inhibitors of USP7.
6) The association of USP7-beta catenin and USP7-p53 has given hope to develop small molecules through peptidomimetics which might prove to be very potential.

Major Objective: Promoting β-Catenin degradation by identifying small molecule through Protein-Protein interaction interface to prevent its interaction from USP7 (the rescue machinery).

Approaches to be:

Objective1: Inhibiting the USP7

- Inhibiting USP7 through small molecules, to prevent deubiquitination from β-catenin, possibly be a strategy to downregulates the Wnt/b-catenin signaling pathway and promote the proteasome-mediated degradation.

Objective2: By targeting the β-catenin pocket directly

- Identification of potential binding site in β-catenin and elucidating the structure-function activity of MSAB (an inhibitor of β-catenin) with β-catenin, which is still unclear.

- Identification of another potent small molecule, which will show much improved activity other than MSAB, by targeting the β-catenin and specifically inhibit β-catenin signaling by promoting its proteasome-mediated degradation and down regulates the Wnt/ β-catenin target genes.

Objective3: By targeting USP7-RNF220 complex or **(USP7-RNF220)-β-catenin complex**

- Predicting the wall of association/protein-protein interface of USP7-RNF220 or (USP7-RNF220)-β-catenin complexes by protein-protein approach.
- These interfaces will help us in designing suitable peptides which will promote β-catenin degradation by disrupting these complexes through peptidomimetics approach.
- Identification of potential binding site in β-catenin where USP7 binds, in order to design peptides of USP7 to bind at β-catenin surface to prevent USP7 binding.
- The identified and validated peptides which are able to block USP7- β-catenin interaction, will be used to generate small molecules through peptidomimetics.
- The identified interface site will be explored at residue level as "hot-spot" and these hot-spot residues will be used for extended multi-step virtual screening process in order to get the known and synthesized active hit molecules.

METHODOLOGY AND TECHNIQUES:

- Molecular Modelling, molecular docking, virtual screening.
- Pharmacophore modeling, Ligand-, structure, fragment-based hit generations and long molecular dynamics simulation studies, Metadynamics and Steered Molecular dynamics.
- Synthesis, Purification and Characterization
- Cell-based in vitro assay systems.

Task/ Quartiles	Year 1				Year 2				Year 3			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Review of Literature												
Model Building and Validation (MD^{apo})												
Molecular Docking												
Interface Study												
Pharmacophore models												
Peptide/inhibitor identification												
$Md^{complex}$												
Validation of MD results												
Experimental Validation												

Table 1: Year-wise work plan

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